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<p>(54) Title: MULTIPLE SITE DELIVERY OF ADENOVIRAL VECTOR FOR THE INDUCTION OF ANGIOGENESIS (57) Abstract. The present invention provides a method for enhancing the level of perfusion of blood to a target tissue, treating a target tissue suffering from or at risk of suffering from ischemic damage, inducing angiogenesis in a target tissue, and/or inducing collateral blood vessel formation in a target tissue affected by or at risk of being affected by a vascular occlusion. The present inventive method comprises administering to the target tissue a dose of a pharmaceutical composition comprising (a) a pharmaceutically acceptable carrier and (b) an adenoviral vector comprising a DNA encoding an angiogenic peptide, such that the level of perfusion of blood to the target tissue is enhanced, the dose has a therapeutic or prophylactic effect on the target tissue, angiogenesis is induced in the target tissue, and/or the adenoviral vector contacts a region including the source, the terminus, and an area therebetween for the collateral blood vessel formation, and collateral blood vessel formation is induced.</p>		

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MULTIPLE SITE DELIVERY OF ADENOVIRAL VECTOR FOR THE INDUCTION OF ANGIOGENESIS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method for
5 enhancing the level of perfusion of blood to a target
tissue, a method for treating a target tissue suffering
from or at risk of suffering from ischemic damage, and a
method of inducing angiogenesis in a target tissue.

10 BACKGROUND OF THE INVENTION

Angiogenesis, the growth of new blood vessels, is a
complex process involving the disruption of vascular
basement membranes, migration and proliferation of
endothelial cells, and subsequent blood vessel formation
15 and maturation. Several mediators are known to elicit
angiogenic responses, and administration of these
mediators promotes revascularization of ischemic tissues.
Vascular endothelial growth factor (VEGF protein) is one
of the most specific of the known angiogenic mediators
20 due to localization of its receptors almost exclusively
on endothelial cells. Receptors for VEGF are upregulated
under ischemic conditions, and the administration of
recombinant VEGF augments the development of collateral
vessels and improves function in peripheral and
25 myocardial ischemic tissue.

However, delivery of VEGF protein remains a
significant challenge. The half-life of VEGF protein is
very short; the administration of high doses of VEGF
protein is associated with hypotension, and systemic
30 administration of VEGF protein can cause promiscuous
induction of angiogenesis in tissues other than that
which has been targeted. Promiscuous induction of
angiogenesis can cause blindness, increase the
aggressiveness of tumor cells, and lead to a multitude of
35 other negative side-effects. Furthermore, the quantity
of VEGF protein delivered is important. If too little
VEGF protein is delivered, angiogenesis will not be

effect on the target tissue. Further provided is a method for inducing angiogenesis in a target tissue comprising administering, via multiple applications to the target tissue, a dose of a pharmaceutical composition comprising (a) a pharmaceutically acceptable carrier and (b) an adenoviral vector comprising a DNA encoding an angiogenic peptide, such that angiogenesis is induced in the target tissue. Additionally provided is a method for inducing collateral blood vessel formation in a target tissue affected by or at risk of being affected by a vascular occlusion comprising administering to the target tissue a dose of a pharmaceutical composition comprising (a) a pharmaceutically acceptable carrier and (b) an adenoviral vector comprising a DNA encoding an angiogenic peptide, such that the adenoviral vector contacts a region including the source, the terminus, and an area therebetween for the collateral blood vessel formation, and collateral blood vessel formation is induced.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention may best be understood with reference to the following detailed description of the preferred embodiments. The present invention provides a method for enhancing the level of perfusion of blood to a target tissue, a method for treating a target tissue suffering from or at risk of suffering from ischemic damage, a method for inducing angiogenesis in a target tissue, and/or a method for inducing collateral blood vessel formation in a target tissue affected by or at risk of being affected by a vascular occlusion. Each of these methods involves administering, via multiple applications to the target tissue, a dose of a pharmaceutical composition comprising (a) a pharmaceutically acceptable carrier and (b) an adenoviral vector comprising a DNA encoding an angiogenic peptide, such that the level of perfusion of blood to the target tissue is enhanced, the dose has a therapeutic or prophylactic effect on the

target tissue, angiogenesis is induced in the target tissue, and/or the adenoviral vector contacts a region including the source, the terminus, and an area therebetween for collateral blood vessel formation, and
5 collateral blood vessel formation is induced.

Induction of Angiogenesis

By the term "inducing angiogenesis," it is meant that angiogenesis is either initiated or enhanced.
10 Therefore, for example, when the target tissue is not already undergoing angiogenesis, the present method provides for the initiation of angiogenesis in the target tissue. However, when the target tissue is already undergoing angiogenesis, the present method provides a
15 means by which the level of angiogenesis is enhanced or heightened.

Target Tissue

Any suitable tissue can be subject to administration
20 within the context of the present invention. Preferably, the target tissue comprises receptors capable of binding the angiogenic peptide encoded by the DNA; more preferably, the target tissue comprises VEGF receptors. Most preferably, the target tissue comprises endothelial
25 cells. Generally, the target tissue will be a part of or form a discrete organ, e.g., a muscle, such as the heart.

Typically, the target tissue will be suffering from or be at risk of suffering from ischemic damage which results when the tissue is deprived of an adequate supply
30 of oxygenated blood. The interruption of the supply of oxygenated blood is often caused by a vascular occlusion. Such vascular occlusion can be caused by arteriosclerosis, trauma, surgical procedures, disease, and/or other indications. There are many ways to
35 determine if a tissue is at risk of suffering ischemic damage from undesirable vascular occlusion. Such methods are well known to physicians who treat such conditions.

For example, in myocardial disease these methods include a variety of imaging techniques (e.g., radiotracer methodologies such as ^{99m}Tc -sestamibi scanning, x-ray, and MRI scanning) and physiological tests. Therefore,
5 induction of angiogenesis in tissue affected by or at risk of being affected by a vascular occlusion is an effective means of preventing and/or attenuating ischemia in such tissue. As a result, although any suitable tissue can be targeted for the induction of angiogenesis,
10 the target tissue is preferably one which is affected by or at risk of being affected by a vascular occlusion.

For example, the blood supply to discrete organs such as the brain, heart, pancreas, entire limbs, or generalized areas of the body, such as a foot, can be
15 attenuated by disease, trauma, surgery, or other events. The alleviation of such attenuated blood supply regardless of its origin is contemplated by the present invention. Thus, prevention or alleviation of damage from indications such as myocardial ischemia and stroke
20 are fully contemplated. Additionally, the planning of a surgical procedure can be predictive of the interruption of blood supply through a particular portion of a patient's vasculature. Prior treatment according to the present method can substantially improve the desired
25 outcome of these surgeries. In that case, treatment preferably occurs about one day to about six weeks before said surgery, and more preferably about two to about fourteen days prior to surgery.

30 Administration of Angiogenic Vector

As previously stated, the induction of angiogenesis via the systemic administration of angiogenic peptides, such as VEGF protein, can lead to promiscuous induction of angiogenesis which, for example, can cause blindness
35 and increase the aggressiveness of tumor cells. Therefore, in order to attenuate or prevent such negative side-effects it is desirable to induce angiogenesis only

in the tissue which requires it (i.e., the target tissue).

The present invention involves the administration of an adenoviral vector comprising a DNA encoding an angiogenic peptide in a localized manner to the target tissue. While any suitable means of administering the angiogenic vector to the target tissue can be used within the context of the present invention, preferably, such a localized administration to the target tissue is accomplished by directly injecting the angiogenic vector into the target tissue or by topically applying the angiogenic vector to the target tissue. By the term "injecting," it is meant that the angiogenic vector is forcefully introduced into the target tissue. Any suitable injection device can be used within the context of the present invention. Such injection devices include, but are not limited to, that described in U.S. Patent Application No. 08/801,352 which is directed to a gene transfer delivery device capable of delivering simultaneous multiple injections. Another example of an injection device which can be used within the context of the present invention includes minimally invasive injection devices. Such devices are capable of accessing the heart, for example, through small incisions of less than 5 inches and are designed to provide injections through a single lumen, in contrast to the multiple injection device described above. To allow for the need for multiple injections with a specific geometry, a marking system can be employed so that the sites of previous injections are well delineated. Minimally invasive injection devices can comprise injector tips which are flexible and steerable to allow access via small incisions to the curved outer surface of the heart, for example, which exists at varying angles with respect to the limited aperture window required with minimally invasive surgeries.

Furthermore, the angiogenic vector can be administered to any suitable surface, either internal or external, of the target tissue. For example, with respect to directly injecting the angiogenic vector into cardiac tissue, it is contemplated that such an injection can be administered from any suitable surface of the heart (i.e., endocardially and/or epicardially). However, it is desirable that whatever means of administering the angiogenic vector is chosen, the induction of angiogenesis in non-targeted tissue is minimized.

While administration of a dose of the angiogenic vector can be accomplished through a single application (e.g., a single injection or a single topical application) to the target tissue, preferably, administration of the dose is via multiple applications of the angiogenic vector. The multiple applications can be 2, 3, 4, 5, or more applications, preferably 5 or more applications, more preferably 8 or more applications, and most preferably at least 10 (e.g., 10, 15, or 20) applications. Multiple applications provide an advantage over single applications in that they can be manipulated by such parameters as a specific geometry defined by the location on the target tissue where each application is administered. The administration of a single dose of the angiogenic vector via multiple applications can be better controlled, and the effectiveness with which any given dose is administered can be maximized. In this way, too, the undesirable effects associated with administration of a single point application of a large dose can be minimized.

The specific geometry of the multiple applications is defined by the location on the target tissue, either in two- or three-dimensional space, where each application of the angiogenic vector is administered. The multiple applications preferably are spaced such that the points of application are separated by up to about 4

cm (e.g., about 0.5-4 cm), more preferably up to about 3 cm (e.g., about 1-3 cm), and most preferably up to about 2 cm (e.g., about 1-2 cm). With respect to the specific geometry of the multiple applications in two-dimensional space, the specific geometry is defined by a plane (i.e., a cross-section of the target tissue) in which the multiple applications lie. The plane defined by the multiple applications can lie at a constant distance from the surface of the target tissue (i.e., substantially parallel to the surface of the target tissue), the depth of the plane, or, alternatively, the plane can lie at an angle with respect to the surface of the target tissue. Preferably, a single application will be administered for about every 0.5-15 cm² of the plane, more preferably for about every 1-12 cm² of the plane, and most preferably for about every 1.5-7 cm² of the plane. The depth of the plane is preferably about 1-10 mm, more preferably about 2-7 mm, and most preferably about 3-5 mm. In three-dimensional space, a single application preferably is administered for up to about 50 cm³ (e.g., about 0.5-50 cm³) of target tissue, more preferably for up to about 35 cm³ (e.g., about 1-35 cm³) of target tissue, and most preferably for up to about 15 cm³ (e.g., about 3-15 cm³) of target tissue. Furthermore, the multiple applications can define any suitable pattern or specific geometry. Therefore, for example, in two-dimensional space, the multiple applications can define a square whereas in three-dimensional space the multiple applications can define a cube.

Another parameter of the multiple applications which can be manipulated is the time differential between each application. Preferably, each of the multiple applications is administered within about 10 minutes (e.g., about 0.5-10 minutes) of each other, more preferably within about 8 minutes (e.g., about 0.5-8 minutes) of each other, and even more preferably within about 6 minutes (e.g., about 1-6 minutes) of each other.

Most preferably, all of the multiple applications of the single dose are administered within the aforesaid time frames. Optimally, each of the multiple applications is administered substantially simultaneously.

- 5 By manipulating both the specific geometry and the time differentials of the multiple applications, the induction of angiogenesis in non-targeted tissue can be minimized.

When administering the angiogenic vector to a target
10 tissue which is affected by or at risk of being affected by a vascular occlusion, it is desirable that the administration is such that the angiogenic vector is able to contact a region reasonably adjacent to the source and the terminus for the collateral blood vessel formation,
15 as well as the area therebetween, which will function as a bypass to the vascular occlusion. It is not believed to be necessary to have the angiogenic vector actually contact the precise sites of the source and the terminus for the collateral blood vessel formation. However,
20 within the context of multiple applications of the angiogenic vector, it is desirable that the specific geometry of the multiple applications be defined to allow the angiogenic vector to contact or reach a region including the source, the terminus, and the area
25 therebetween for the collateral blood vessel formation, preferably to actually contact the precise sites of the source and the terminus for the collateral blood vessel formation, along with the area therebetween.

Furthermore, administration of the angiogenic vector
30 to the target tissue can be accomplished either *in vivo* or *ex vivo*. Therefore, for example, the target tissue can be removed from the recipient of the present inventive method, can be treated with the angiogenic substance, and then can be reimplanted into the
35 recipient. *Ex vivo* administration of the angiogenic substance to the target tissue also helps to minimize

undesirable induction of angiogenesis in non-targeted tissue.

Angiogenic Vector

5 As previously stated, the delivery of VEGF protein as an angiogenic substance to tissue remains a significant challenge due, in large part, to its very short half-life. However, by utilizing an adenoviral vector comprising a DNA encoding an angiogenic peptide as
10 the angiogenic substance, it is possible to infect host cells and thereby induce the sustained, predictable, and effective production of an angiogenic peptide for about a week. After about a week, the adenoviral vector ceases to produce the angiogenic peptide and, to that extent,
15 the present invention provides a self-terminating method of inducing angiogenesis.

Adenoviral vectors are preferred because, unlike plasmids and other viral vectors (e.g., herpes simplex virus), adenoviral vectors achieve gene transfer in both
20 dividing and nondividing cells, with high levels of protein expression in cardiovascular relevant sites such as myocardium, vascular endothelium, and skeletal muscle. Furthermore, the gene transferred by an adenoviral vector functions in an epi-chromosomal position and thus carries
25 little risk of inappropriately inserting the transferred gene into a critical site of the host genome. The adenoviral vector also is preferably deficient in at least one gene function required for viral replication. Preferably, the adenoviral vector is deficient in at
30 least one essential gene function of the E1 region of the adenoviral genome, particularly the E1a region, more preferably, the vector is deficient in at least one essential gene function of the E1 region and part of the E3 region (e.g., an XbaI deletion of the E3 region) or,
35 alternatively, the vector is deficient in at least one essential gene function of the E1 region and at least one essential gene function of the E4 region. However,

adenoviral vectors deficient in at least one essential gene function of the E2a region and adenoviral vectors deficient in all of the E3 region also are contemplated here and are well known in the art. Adenoviral vectors deleted of the entire E4 region can elicit lower host immune responses. Suitable replication deficient adenoviral vectors are disclosed in PCT International Publication Nos. WO 95/34671 and WO 97/21826. For example, suitable replication deficient adenoviral vectors include those with a partial deletion of the E1a region, a partial deletion of the E1b region, a partial deletion of the E2a region, and a partial deletion of the E3 region. Alternatively, the replication deficient adenoviral vector can have a deletion of the E1 region, a partial deletion of the E3 region, and a partial deletion of the E4 region.

Furthermore, the viral vector's coat protein can be modified so as to incorporate a specific protein binding sequence, as described in PCT International Publication No. WO 96/2628, or the viral vector's coat protein can be modified so as to decrease the viral vector's ability or inability to be recognized by a neutralizing antibody directed against the wild-type coat protein, as described in U.S. Patent Application Serial No. 08/816,346.

Any DNA encoding an angiogenic peptide and operably linked to suitable expression signals can be used within the context of the present invention. Whereas the DNA can be operably linked to any suitable set of expression signals, preferably, the expression of the DNA is under the control of the cytomegalovirus (CMV) immediate early promoter.

Additionally, the DNA can encode any suitable angiogenic peptide. Preferably, the angiogenic peptide is a VEGF protein, and more preferably, the angiogenic peptide is VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, or a mammalian counterpart, which are variously described in U.S. Patents 5,332,671 (Ferrara et al.), 5,240,848 (Keck et

al.), and 5,219,739 (Tischer et al.). Most preferably, because of their higher biological activity, the angiogenic peptide is VEGF₁₂₁ or VEGF₁₆₅, particularly VEGF₁₂₁. A notable difference between VEGF₁₂₁ and VEGF₁₆₅ is
5 that VEGF₁₂₁ does not bind to heparin with a high degree of affinity as does VEGF₁₆₅. Generally, VEGF moieties are advantageous over other angiogenic peptides because VEGF proteins do not induce the growth of tissues not involved in the production of new vasculature. Other angiogenic
10 peptides include VEGF II, VEGF-C, FGF-4, angiogenin, angiogenin-2, and PlGF, which are variously described in U.S. Patents 5,338,840 (Bayne et al.) and 5,532,343 (Bayne et al.), International Patent Application WO
95/24473 (Hu et al.), European Patent Documents 476 983
15 (Bayne et al.), 506 477 (Bayne et al.), and 550 296 (Sudo et al.), and Japanese Patent Documents 1038100, 2117698, 2279698, and 3178996.

The adenoviral vector also can include a DNA encoding an angiogenic peptide receptor. Suitable
20 angiogenic peptide receptors include, for example, FLT-1, FLK-1, and FLT-4. Indeed, in certain embodiments, the adenoviral vector can utilize a DNA encoding an angiogenic peptide receptor in place of, rather than in addition to, the DNA encoding an angiogenic peptide.

25 The DNA, operably linked to expression signals and encoding the angiogenic peptide, can be inserted into any suitable region of the adenoviral vector as an expression cassette. In that respect, the skilled artisan will readily appreciate that there are certain advantages to
30 using an adenoviral vector deficient in some essential gene region of the adenoviral genome inasmuch as such a deficiency will provide room in the vector for a transgene and will prevent the virus from replicating. Preferably, the DNA segment is inserted into the E1
35 region of the adenoviral vector. Whereas the DNA segment can be inserted as an expression cassette in any suitable orientation in any suitable region of the adenoviral

vector, preferably, the orientation of the DNA segment is from right to left. By the expression cassette having an orientation from right to left, it is meant that the direction of transcription of the expression cassette is opposite that of the region of the adenoviral vector into which the expression cassette is inserted.

An adenoviral vector illustrative of the present inventive vector is deficient in the E1a region, part of the E1b region, and part of the E3 region of the adenoviral genome and contains the DNA encoding human VEGF₁₂₁ or human VEGF₁₆₅ under the control of the CMV immediate early promoter in the E1 region of the adenoviral genome. Such a vector supports *in vivo* expression of VEGF that is maximized at one day following administration and is not detectable above baseline levels as little as one week after administration. This is ideal inasmuch as it is sufficient to provide substantial growth of new vasculature while minimizing adverse neovascularization at distal sites. In that regard, when this vector is locally administered to a target tissue, no detectable VEGF expression can be detected in blood serum using standard ELISA monitoring assays.

Advantageously, local administration to a target tissue of adenoviral vectors encoding human VEGF₁₂₁ or VEGF₁₆₅ in the E1 region of the adenoviral genome are able to increase blood flow at least 3-fold in the extremities of mammals (e.g., the hindlimb of Sprague-Dawley rats) with iliac and femoral artery ligations.

Pharmaceutical Composition

The angiogenic vector desirably is administered to the target tissue in a pharmaceutical composition which comprises a pharmaceutically acceptable carrier and the angiogenic vector.

Any suitable pharmaceutically acceptable carrier can be used within the context of the present invention, and

such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition is to be administered and the particular method used to administer the composition.

5 Formulations suitable for injection include aqueous and non-aqueous solutions, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and

10 aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored

15 in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind

20 previously described. Preferably, the pharmaceutically acceptable carrier is a buffered saline solution.

Although any suitable volume of carrier can be utilized within the context of the present invention, preferably, the angiogenic vector is administered in

25 small volumes of carrier so that the tissue to be vascularized (i.e., the target tissue) is perfused with the angiogenic vector but the angiogenic vector is not carried by the blood, lymphatic drainage, or physical mechanisms (e.g., gravitational flow or osmotic flow) to

30 tissues which have not been targeted.

In the case of most applications, particularly to discrete organs such as with respect to human myocardial injections, the volume administered is preferably less than 20 ml (e.g., about 0.1-20 ml) per each

35 administration and more preferably less than about 2.5 ml (e.g., about 0.5-2.5 ml) per each administration.

Dosage

The determination of the proper dosage of the angiogenic vector can be easily made by those of ordinary skill in the art. However, generally, certain factors
5 will impact the dosage which is administered.

Although the proper dosage is such that angiogenesis is induced in the target tissue, preferably, the dosage is sufficient to have a therapeutic and/or prophylactic effect on target tissue which is affected by or at risk
10 of being affected by a vascular occlusion which may lead to ischemic damage of the tissue. Additionally, the dosage should be such that induction of angiogenesis in non-targeted tissue is minimized.

The dosage also will vary depending upon the
15 angiogenic substance to be administered. Specifically, the dosage will vary depending upon the particular vector and DNA, encoding and controlling the expression of the angiogenic peptide in the vector, which are utilized. A dose typically will be at least about 1×10^6 pfu (e.g.,
20 1×10^6 - 1×10^{13} pfu) to the target tissue, e.g., a discrete organ, such as a human heart. The dose preferably is at least about 1×10^7 pfu (e.g., about 1×10^7 - 1×10^{13} pfu), more preferably at least about 1×10^8 pfu (e.g., about 1×10^8 -
25 1×10^{13} pfu), and most preferably at least about 1×10^9 pfu (e.g., about 1×10^9 - 1×10^{10} pfu). The dose typically is for a volume of targeted tissue of about 100 cm^3 , more typically about 150 cm^3 . The dose is administered via
multiple applications, and, as such, is divided among the multiple applications. Thus, if the dose is administered
30 via 10 administrations, each administration involves about 1×10^5 - 1×10^{12} pfu. Preferably, each application involves about 1×10^6 - 1×10^{12} pfu, more preferably about 1×10^7 - 1×10^{10} pfu, and most preferably about 1×10^8 - 1×10^9 pfu. For purposes of considering the dose in terms of
35 particle units (pu), also referred to as viral particles, it can be assumed that there are 100 particles/pfu (e.g., 1×10^{12} pfu is equivalent to 1×10^{14} pu). In a single round

of vector administration, using, for example, an adenoviral vector deleted of the Ela region, part of the Elb region, and part of the E3 region of the adenoviral genome, wherein the vector carries human VEGF₁₂₁ or VEGF₁₆₅ under the control of a standard CMV immediate early promoter, about 10^7 - 10^{11} pfu, preferably about 10^9 - 10^{11} pfu, are administered to a targeted tissue (e.g., to a discrete organ containing the targeted tissue) with an estimated volume of about 150 cm³. Under these conditions, a substantial level of VEGF production is achieved in the target tissue without producing detectable levels of VEGF production in distal tissues.

Furthermore, with respect to multiple applications of the angiogenic vector, each application can be such that a dosage gradient is administered across the region defined by the multiple applications. Alternatively, each of the multiple applications can be such that a substantially uniform dose is administered across the region defined by the multiple applications.

EXAMPLES

The following examples further illustrate the present invention but in no way should be construed to limit the scope thereof.

Example 1

This example illustrates the present invention's ability to induce angiogenesis *in vivo* by administration of a replication-deficient recombinant adenovirus vector (Ad vector). Comprising a DNA encoding an angiogenic (particularly VEGF) peptide.

The replication-deficient recombinant Ad vector containing the DNA for an illustrative angiogenic (particularly VEGF peptide) VEGF₁₆₅, was engineered according to a technique described in Gastroenterology, 106, 1638-1644 (1994). The DNA for VEGF₁₆₅, including the signal sequence for secretion, was inserted into an

expression plasmid and was under the control of the constitutive CMV immediate-early promoter/enhancer. The expression plasmid also contained the Ad5 sequence from nucleotide 3384 to nucleotide 5778 (9.24 to 16.05 map units), which served as the homologous recombination sequence. The plasmid carrying the DNA for VEGF₁₆₅ was cotransfected with the plasmid pJM17 (from F. Graham, McMaster University, Hamilton, Ontario, Canada) into 293 cells (American Type Culture Collection, CRL1573). The plasmid pJM17 contains the full-length Ad5 DNA (36 kb) and pBRX, a 4.2-kb insert placed in the E1 region, thus exceeding by approximately 2 kb the maximum packaging limit of DNA into the Ad capsid. Homologous recombination between the expression plasmid and pJM17 in 293 cells replaced the E1 region and pBRX insert with the expression cassette from the expression plasmid. The growth of E1 deleted adenoviral vectors is limited to complementary cells, and was carried out in 293 cells, a human embryonic kidney cell line that has been transformed by Ad5 and expresses the E1 region in trans. Culture medium for the 293 cells was improved minimal essential medium with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (all from Biofluids). After cotransfection, individual viral plaques were isolated and amplified in 293 cells. The control vector was AdCMV.βgal, which carries the DNA for the E. coli lacZ gene and codes for the enzyme β-galactosidase. AdCMV.VEGF₁₆₅ and AdCMV.βgal were propagated in 293 cells and were purified by CsCl density purification. Subsequently, the preparations were dialyzed and stored in dialysis buffer (10 mmol/l Tris-HCl and 1 mmol/l MgCl₂, pH=7.4) with 10% glycerol at -70 °C. The titer of each viral stock was determined by plaque assay in 293 cells, and the titers consistently ranged between 5x10⁷ and 2x10¹¹ pfu/ml.

In order to assess the effects of Ad-mediated gene transfer in vivo, either AdCMV.VEGF₁₆₅ or AdCMV.βgal (2x10¹⁰ pfu) was resuspended in 0.5 ml Matrigel. Subsequently, C57BL mice (Jackson Laboratories, Bar Harbor, ME) were injected subcutaneously, near the abdominal midline, with the entire 0.5 ml Matrigel containing either AdCMV.VEGF₁₆₅ or AdCMV.βgal. Additional animals were injected with vector-free Matrigel. Mice were studied according to four different protocols.

Protocol 1: To establish whether Ad vectors resuspended in Matrigel infect the surrounding tissues, mice were injected either with Matrigel containing AdCMV.βgal (n=5) or Matrigel alone (n=3). The animals were killed 6 days after injection, and the Matrigel plugs were removed and fixed. Subsequently, the Matrigel plugs were sectioned, stained with X-gal, and examined for evidence of blue staining.

Protocol 2: To establish the duration of transgene expression in vivo, mice were injected either with Matrigel containing AdCMV.VEGF₁₆₅ (n=9), or Matrigel alone (n=9). Animals were killed, and the Matrigel plugs were removed 3, 7, and 21 days after injection. Tissue blocks were immersed in OCT compound (Miles, Inc.) and rapidly frozen in liquid nitrogen. Tissue blocks were stored at -70 °C for less than 1 month. For immunohistochemical evaluation, 10 μm frozen sections (Microm cryotome) were mounted on silanated slides (Digene Diagnostics). Sections were air-dried for 15 minutes, and either stored at -70 °C for up to 48 hours or fixed immediately in 1x Histochoice (Amresco) containing 0.1% Triton X-100 (Sigma Chemical Co.) for 12 minutes. After they were washed with PBS (ph=7.4), slides were incubated in 0.5% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity. Anti-VEGF primary rabbit antibodies were detected by using biotinylated goat anti-rabbit IgG secondary antibody and the avidin-biotin complex and visualized by diaminobenzidine (all detection reagents

were from Vector Laboratories). Procedures were performed according to package directions, except sections were kept in blocking solution for at least 45 minutes before the addition of the primary antibody, and
5 incubations with anti-VEGF or control serum (1:6000 dilution) were performed overnight at 4 °C. Sections were counterstained in hematoxylin. Anti-VEGF antibodies were produced in rabbits, except the peptide was conjugated to a carrier protein, KLH, by 0.2% glutaraldehyde.
10 Antibodies to KLH alone were also raised and used as a negative control. Antibody specificity was determined by recognizing human VEGF on Western blots, and both anti-KLH and prebleed serum were used as negative controls to determine background staining.

15 Protocol 3: The presence of newly formed blood vessels was evaluated, as described in Lab Invest., 67, 519-528 (1992), in mice killed 14 days after the injection of the Matrigel (n=8 mice for each Ad vector; 4 mice were used in each of two separate experiments). The
20 gels were recovered by dissection and fixed. Histological sections were stained with Masson's trichrome stain and evaluated for the presence of neovascularization. The thickness of the stroma surrounding the Matrigel was assessed by measuring the
25 distance between the surface of the Matrigel and the abdominal muscle in two different histological sections from each plug. Ten measurements were obtained at 50 to 100 µm intervals from each histological section, and the 20 measurements from the two sections were averaged to
30 express stromal thickness for each individual plug.

Protocol 4: The angiogenic response was quantified by the hemoglobin content of the Matrigel plugs (n=10 mice for each Ad vector; 3 or 4 mice were used in each of three separate experiments).

35 With respect to the experiments conducted to establish whether Ad vectors resuspended in Matrigel could diffuse out of the gel and infect the surrounding

tissue, mice were killed 6 days after injection of Matrigel containing AdCMV. β gal or Matrigel alone, and the Matrigel plugs were stained with X-gal. X-gal positive cells were found in the stroma surrounding the Matrigel.

5 In contrast, no blue cells were found in the tissue surrounding uninfected gel plugs. In other experiments, the duration of Ad-mediated VEGF₁₆₅ gene expression *in vivo* was established. By immunohistochemical staining, plugs recovered 3 days after coinjection of Matrigel and

10 AdCMV.VEGF₁₆₅ showed VEGF-positive cells in the tissue surrounding the Matrigel. Staining was most intense at day 7, and only a few cells were immunoreactive 21 days after injection. Incubations in the absence of the primary antibody showed no immunostaining. Incubations

15 with the antibody against the carrier protein showed positivity in the abdominal muscle layer; however, no positivity was found in the tissue surrounding the Matrigel plugs. The Matrigel plugs were examined histologically 14 days after injection, and angiogenesis

20 was observed in the tissues surrounding the Matrigel in response to AdCMV.VEGF₁₆₅. This effect was associated with increased vascularity and thickening of the stromal matrix surrounding the Matrigel. In contrast, AdCMV. β gal resulted in some thickening of the stromal matrix

25 surrounding the Matrigel without evidence of increased vascularization, and Matrigel alone was not associated with increased stromal thickening or angiogenesis. Furthermore, the quantitative assessment of angiogenesis demonstrated that the hemoglobin content of the Matrigel

30 plugs with AdCMV.VEGF₁₆₅ was four-fold higher than in the case of the gel explants with AdCMV. β gal. A significant increase in hemoglobin content also was observed with AdCMV. β gal-infected versus uninfected control plugs. Together, these results show that an adenoviral vector

35 comprising a DNA encoding an angiogenic (particularly VEGF) peptide induces angiogenesis *in vivo*.

Example 2

This example illustrates the present invention's ability to direct in vivo gene transfer to myocardium using a replication-deficient adenovirus vector.

5 A replication-deficient vector, AdCMV.VEGF, was an Ela', partial Elb', partial E3' adenovirus vector that contained an expression cassette in the E1 position which contained the cytomegalovirus immediate early promoter/enhancer (CMV) driving the DNA for human VEGF₁₆₅.
10 AdCMV.Null (similar to AdCMV.VEGF, but with no gene in the expression cassette) was used as a control vector for in vitro experiments. AdCMV.CAT (similar to AdCMV.VEGF, but coding for chloramphenicol acetyl transferase) was used to transfer and express a marker gene. All
15 adenovirus vectors were propagated in 293 cells, purified by CsCl density purification, dialyzed, and titered by plaque assay. Vectors were stored in 50 µl aliquots at -70 °C.

20 Male mongrel dogs (25 to 30 kg) were used for all the studies. Anesthesia was induced with intravenous methohexital (Brevital; Eli Lilly, Indianapolis, Indiana; 10 mg/kg), and after intubation, anesthesia was maintained using inhaled isoflurane (1- 2% in 2-3 l O₂). For direct myocardial injections, a left lateral
25 thoracotomy was performed under sterile conditions. The pericardium was divided anterior to the phrenic nerve, and three separate marking sutures (5-0 monofilament) were placed at 3.5 cm intervals along the left ventricular free wall. Adenovirus vectors were
30 administered at marked locations in a volume of 100 µl using a 0.5 ml syringe with a 30 gauge needle. The needle tip was positioned at a depth of 3 to 5 mm from the epicardial surface, and satisfactory delivery was confirmed visually. The pericardium and chest were
35 closed in a standard fashion, and the animals were permitted to recover.

To evaluate the feasibility of achieving sustained local levels of a therapeutic angiogenic protein in myocardium, AdCMV.VEGF (10^5 pfu) was administered by direct myocardial injection (two injections per animal; 5 12 animals). Tissue samples (1 cm³) from the site of vector administration were harvested and evaluated for VEGF expression immediately and at 2, 5, 7, and 14 days after vector administration. Tissue injected with the AdCMV.CAT vector was used as a negative control.

10 Quantification of VEGF expression in myocardium was performed with the Quantikine human VEGF immunoassay (R&D Systems, Inc., Minneapolis, MN). Tissue samples (0.5 g) from the sites of vector administration were homogenized with protein lysis buffer (10 mmol/l Tris-HCl, pH=8, 0.14 15 mol/l NaCl, 0.025% NaN₃, 2% Triton X-100, and 1 mmol/l phenylmethylsulfonyl fluoride; 2 ml/g tissue), protein determinations were performed, aliquots of protein lysate (100 µg) were analyzed in triplicate, and absorbance was measured at 450 nm using a microplate reader. The 20 concentration of VEGF was normalized to mg protein. The spatial limit of VEGF expression was determined by evaluation of tissue samples from animals sacrificed at 7 days. Tissue was divided into central, peripheral, epicardial, and endocardial components, and each sample 25 was evaluated individually for VEGF expression.

To determine whether localized expression of VEGF would result in detectable levels of VEGF in the serum, blood samples were obtained from the animals before vector administration and at the times of sacrifice at 2, 30 5, 7, and 14 days after vector administration. Quantification of VEGF was performed by enzyme-linked immunosorbent assay on 50 µl samples of serum.

To evaluate the systemic effect of direct myocardial injections of adenoviral vectors in the myocardium, serum 35 biochemistry and complete blood count indices were monitored over time. Blood samples for white blood cell count, hematocrit, platelet count, alkaline phosphatase,

serum glutamic-pyruvic transaminase, bilirubin, and creatinine were obtained from the animals before vector administration and at 2, 7, and 14 days after vector administration. Values for each time were averaged.

- 5 Serum chemistry determinations were calculated with the Du Pont Analyst Benchtop Chemistry System (Du Pont Co., Wilmington, DE), and complete blood count determinations were made with the System 900 Hematology Analyzer (Serono Diagnostics, Allentown, PA).

- 10 To determine the effect of direct myocardial gene transfer on cardiac function, transthoracic two-dimensional Doppler and echocardiograms using a Hewlett-Packard 2500 echocardiographic machine (Hewlett-Packard Co., Andover, MA) and a 3.5-MHz transducer were
15 performed. The following images were obtained preoperatively and either 5 to 7 days postoperatively or 14 days postoperatively: the parasternal long-axis view, the parasternal short-axis view at the tip of the papillary muscles, and the apical five-chamber view.
20 Pulsed-wave Doppler echocardiography at the level of the aortic annulus was also performed from the apical five-chamber view.

- Off-line analysis of regional wall thickening was performed by tracing the endocardial and epicardial
25 surfaces of the left ventricle in both diastole and systole. The ventricle was then divided into six equal radial segments, with segment 1 beginning at the inferior ventricular septum and subsequent segments labeled consecutively in a clockwise fashion, with segment 6
30 ending at the inferior wall. Segments 3 and 4, therefore, represent the anterolateral free wall of the left ventricle. The mean wall thickness of each segment was determined. Systolic wall thickening in each segment was defined as the mean systolic wall thickness minus the
35 mean diastolic wall thickness. As an assessment of global left ventricular function, cardiac output was calculated using standard Doppler-derived stroke volume

(aortic annular area times the velocity time integral of the flow velocity profile across the aortic annulus) and the recorded heart rate.

- Confirmation of VEGF protein expression *in vivo* after direct myocardial administration of AdCMV.VEGF was achieved by enzyme-linked immunosorbent assay. Quantification of VEGF expression in myocardium over time demonstrated that administration of AdCMV.VEGF resulted in a more than 18-fold increase over baseline VEGF expression 2 days after vector administration and a more than 15-fold increase over baseline 7 days after vector administration. By day 14, VEGF levels had returned to baseline. In contrast, administration of the control vector AdCMV.CAT did not result in significantly increased levels of VEGF expression over baseline at any of the times examined. The levels of VEGF on day 0, obtained immediately after vector administration, were similar to levels in the naive tissue, confirming that the viral preparation was not contaminated with VEGF protein. Furthermore, in support of the concept that adenovirus vector delivery provides a localized gene transfer strategy, no levels of VEGF could be detected in the serum of treated animals at any of the times examined.
- Consistent with the data obtained with marker gene transfer, the administration of AdCMV.VEGF was also characterized by a wide spatial limit of gene expression. In contrast to the results obtained for AdCMV.CAT, however, 7 days after administration of AdCMV.VEGF, levels of VEGF expression were equally increased in all four spatial regions (central and peripheral epicardium and central and peripheral endocardium) up to 15 mm from the site of vector administration, suggesting a homogeneous distribution of protein through all tissue samples.

All animals that received adenovirus vectors survived to their predetermined times for sacrifice. No

animals demonstrated failure to thrive or tachycardia or were febrile, and no wound infections developed. There were no significant changes over baseline in white blood cell count, hematocrit, platelet count, alkaline
5 phosphatase, serum glutamic-pyruvic transaminase, bilirubin, or creatinine in the animals examined at 2, 7, and 14 days after vector administration.

Echocardiograms performed on the animals before vector administration and 5 to 7 days or 14 days after
10 administration demonstrated no significant change in global or regional ventricular function. Regional wall motion assessment revealed no significant difference in the systolic wall thickening of any of the six radial segments between the preoperative and postoperative
15 studies. Cardiac output also did not change significantly between the preoperative and postoperative studies.

In summary, it has been shown that a wide spatial limit of gene expression has been characterized after
20 adenovirus-mediated direct myocardial gene transfer, and the delivery system has been shown to be safe and well tolerated. Furthermore, using a large animal model with physiology similar to that of a human, it has been shown that an in vivo administration of an adenovirus vector
25 encoding a therapeutic angiogenic protein (particularly VEGF) resulted in sustained and localized protein expression for a number of days after gene transfer.

Example 3

30 This example illustrates the present invention's ability to protect against threatened ischemic vascular occlusions by inducing salvage angiogenesis via adenoviral-mediated gene transfer of an angiogenic peptide (particularly VEGF).

35 A model of acute vascular occlusion superimposed upon pre-existing ischemia was created using male Sprague-Dawley rats weighing 250 to 300 g. Animals were

anesthetized with intramuscular ketamine (100 mg/kg) and xylazine (2 mg/kg), a midline laparotomy was performed under sterile conditions, and the left common iliac artery was ligated and divided. The adenoviral vectors were then administered in the left iliofemoral adipose tissue and muscle, and the abdomen was closed in two layers with non-absorbable suture. AdVEGF (total dose= 4×10^9 pfu), the control vector AdNull (total dose= 4×10^9 pfu), or PBS (total dose= $4 \times 100 \mu\text{l}$) at the time of iliac artery ligation was administered in a volume of 100 μl at each of four sites using a 0.5 ml syringe with a 30-gauge needle. In the region targeted for collateral vessel formation, 4 individual vector administrations were performed at consistent locations in the left iliofemoral region of each animal, including retroperitoneal and inguinal adipose tissue, psoas muscle, and quadriceps muscle. An additional group of control animals underwent unilateral common iliac ligation alone with no treatment.

Three weeks following left common iliac ligation and vector administration, animals were anesthetized as described above, and the left common femoral artery was ligated and divided at the level of the inguinal ligament. Immediate analysis of relative hindlimb blood flow and vascularity were then conducted utilizing, in the following order: (1) $^{99\text{m}}\text{Tc}$ -labeled sestamibi; (2) color microspheres; (3) angiography; and (4) histologic quantification of blood vessel number.

The replication-deficient vector AdVEGF was an E1a⁻, partial E1b⁻, partial E3⁻ adenoviral vector that contained an expression cassette in the E1 position containing the cytomegalovirus immediate early promoter/enhancer (CMV) driving the DNA for the 165 residue form of human VEGF (VEGF₁₆₅). AdNull (similar to AdVEGF, but with no gene in the expression cassette) was used as a control vector. All adenoviral vectors were propagated in 293 cells, purified by CsCl density purification, dialyzed, and

stored at -70 °C. The titer of each viral stock was determined by plaque assay in 293 cells. All viral stocks were demonstrated to be free of replication-competent wild-type adenovirus.

5 To confirm that the AdVEGF vector could mediate transfer and expression of the VEGF DNA to adipose tissue and skeletal muscle, an enzyme-linked immunoassay (ELISA) was used to quantify VEGF levels in tissue recovered from animals 0, 1, 3, 5, and 7 days following common iliac
10 ligation and vector administration (10^8 pfu/site; n=3 each time point). Retroperitoneal adipose tissue and quadriceps muscle of animals treated with the vectors were harvested, rinsed in phosphate-buffered saline, pH=7.4 (PBS), and homogenized with protein lysis buffer
15 [10 mM Tris-HCl pH=8, 0.14 M NaCl, 0.025% NaN₃, 2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (2 ml/g tissue)]. To confirm that the VEGF produced remained localized, serum was obtained from each animal at the above time points. Protein determinations were performed
20 using the Bradford method, and ELISA for VEGF was carried out [Quantikine human VEGF Immunoassay, R&D Systems, Minneapolis, MN] using 50 µg tissue (each assay carried out in duplicate for each animal). Absorbance was measured at 450 nm using a microplate reader, and VEGF
25 concentration was normalized to mg protein.

^{99m}Tc-labeled sestamibi scanning was used as a measure of blood flow to the hindlimb. To accomplish this, the right jugular vein was identified through a limited right cervical incision, and 2-3 mCi of ^{99m}Tc-
30 labeled sestamibi (Cardiolite, Dupont Pharma, North Billerica, MD) in a volume of 0.5 ml of PBS (0.9%) was injected intravenously. Approximately 15 min after the injection, animals were placed in the supine position on the lower detector of an ADAC Vertex dual head gamma
35 camera system (ADAC Laboratories, Milpitas, CA), and ventral and dorsal whole-body gamma camera images were acquired using low-energy high-resolution parallel-hole

collimators and a photopeak energy window of $140 \text{ keV} \pm 10\%$. At least 2×10^5 counts per animal were acquired simultaneously from the dorsal (lower detector) and ventral (upper detector) images. Pegasys™ computer and image processing software (ADAC Laboratories) were used to manually draw consistent rectangular regions of interest (ROI) over the center of the calves of the left and right hindlimbs by an individual blinded to the treatment groups, and the mean counts per pixel in these regions were determined. Relative blood flow was reported as the ratio of the ROI mean counts per pixel in the ligated (left) hindlimb to the ROI mean counts per pixel in the contralateral (right) control hindlimb for the geometric mean of ventral and dorsal images.

Blood flow to the ischemic (left) hindlimb relative to the normal (right) hindlimb also was evaluated by intraarterial administration of $15 \mu\text{m}$ color microspheres as an assessment of functional blood vessels to the limb. The abdominal aorta was identified through a midline laparotomy, and loosely encircled with a 4-0 silk suture. Immediately distal to the suture, a 24-gauge, 3/4-inch (1.9 cm) Angiocath (Becton Dickinson Vascular Access, Sandy, UT) was inserted into the infrarenal aorta, and 0.5 ml of a solution of nitroglycerin (Abbott Laboratories, North Chicago, IL ($500 \mu\text{g/ml}$)) and 2×10^6 of $15 \mu\text{m}$ color microspheres [E-Z Trac, Los Angeles, CA (2×10^6 microspheres in $200 \mu\text{l}$)] were vortexed and injected through the catheter over 20 seconds to ensure adequate mixing of the microspheres. At sacrifice, all lower hindlimb calf musculature was dissected free from skin and bone, weighed, digested with Tissue Digest Reagents 1 and 2 (E-Z Trac) according to manufacturer's instructions, and resuspended in $50 \mu\text{l}$ Microsphere Counting Reagent (E-Z Trac). Microspheres were counted by an individual blinded to treatment groups using a manual hemocytometer, with a minimum of 400 spheres counted per sample. The number of microspheres per gram

of tissue was determined, and relative blood flow was reported as microspheres/gm (wet weight) tissue in the ligated hindlimb versus microspheres/gm of wet tissue in the contralateral control hindlimb.

- 5 Angiography was used as an assessment of macroscopic vascularity. To do this, animals were positioned supine, at a distance of 20 cm from the collimator of a Mobile Surgical X-ray System BV25 (Philips, Holland). Using a 24 gauge, 3/4-inch (1.9 cm) angiocatheter (Becton
10 Dickinson, Sandy, UT) placed into the infrarenal aorta, 0.5 ml nitroglycerin (500 µg/ml; Abbott Laboratories) was injected over 20 seconds. Immediately thereafter, 3 ml of Renograffin-76 (Squibb Diagnostics, New Brunswick, NJ) were injected through the catheter into the distal aorta,
15 and fluoroscopic images were obtained at 2 second intervals. Representative images demonstrating maximal arterial opacification were developed, and vascularity was scored by three observers in a blinded fashion. A vascular score was determined for each animal by drawing
20 a line perpendicular to the midpoint of the long axis of the femur; the number of vessels crossing this line were counted by each observer, averaged, and reported as a "vascular score."

- Histologic evaluation was used to quantify
25 vascularity at the small vessel level at the sites receiving the adenoviral vectors. For the treated adipose tissue sites, 1 cm³ adipose tissue samples from sites of vector administration were retrieved, rinsed in PBS and stored in 4% formalin at 4 °C. Samples were
30 embedded in paraffin, serial 5 µm cross-sections in a plane parallel to the surface of the tissue were obtained at intervals of 50 µm, and immunohistochemical staining for α-actin, an endothelial cell specific antigen, was performed. Paraffin sections were blocked with 1.5%
35 horse serum for 20 minutes to prevent non-specific binding, and then exposed to primary antibody (monoclonal anti-human α-actin; Sigma, St. Louis, MO) at a dilution

of 1/500 for 60 minutes. The slides were exposed sequentially (30 minutes each) to biotinylated horse anti-mouse IgG, ABC reagents (Vector Laboratories, Burlingame, CO), new fuchsin substrate for alkaline phosphatase (Dako Corp., Carpinteria, CA), and then counterstained with hematoxylin. Sections were examined in a blinded fashion by 3 observers at a magnification of 100x. Five random fields of vessels less than 80 μ m were counted per slide; 6 slides were evaluated per sample.

10 The counts were averaged and reported as number of blood vessels per mm².

To quantify vascularity in treated regions of skeletal muscle, 1 cm³ sections of quadriceps muscle from sites of vector administration were retrieved, rinsed in PBS and then fixed in increasing concentrations of a sucrose phosphate solution (25 °C, 1 hour). The skeletal muscle specimens were then frozen in a 2:1 20% sucrose/OCT (Tissue Tek, Sakura Finetek, Torrance, CA) compound mixture at -70 °C. The frozen specimens were then cut into 5 μ m sections and frozen on the slides at -70 °C. The frozen slides were warmed to room temperature, stained for alkaline phosphatase (25 °C, 1 hour) using an insoluble alkaline phosphatase substrate-5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium (BCIP/NBT tablets, Sigma, St. Louis, MO) and counterstained with eosin. Sections were examined at a magnification of 400x in a blinded fashion by 3 observers. Capillaries per muscle fiber were quantified by counting 6 random muscle fibers per field, 5 random fields per slide and 5 slides per animal. The counts were averaged and reported as number of capillaries per muscle fiber.

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Successful AdVEGF-mediated gene transfer and expression in adipose and skeletal muscle was confirmed by ELISA. There was no detectable VEGF in either tissue on day 0 (obtained immediately following vector administration), similar to naive adipose tissue and

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skeletal muscle, confirming that the vector preparation was not contaminated with VEGF protein. For both tissues, quantification of VEGF expression demonstrated that administration of AdVEGF resulted in VEGF expression, peaking 1 day following vector administration, and decreasing over several days to baseline by 1 week following vector administration. In contrast, administration of the control vector AdNull resulted in no detectable VEGF expression in either tissue at any timepoint. Importantly, consistent with the concept that the adenoviral vector delivery provides a localized gene transfer and expression strategy, no VEGF could be detected in the serum of AdVEGF-treated animals at any of the timepoints after vector delivery, and administration of AdNull did not result in increased serum levels of VEGF over baseline. Endogenous VEGF could not be detected in serum or in the tissues as the ELISA used for these experiments detects human VEGF only and not rat VEGF.

^{99m}Tc-labeled sestamibi imaging demonstrated a significantly greater relative blood flow to the ischemic hindlimb in animals treated with AdVEGF. The scanned radioactive images of AdNull-treated control animals demonstrated low level of radioactivity in the calf region of the ligated hindlimb. Naïve and PBS-treated control animals had similar low levels of radioactivity in this region, as did naïve and AdNull controls. Although both the AdNull and PBS controls had low levels of radioactivity in the calf region, the AdNull group was slightly higher than the PBS group.

Color microsphere analysis demonstrated that the relative blood flow following femoral artery ligation in AdVEGF-treated animals was nearly 3-fold greater than that observed in any of the control animals [AdNull-treated, PBS-treated or naïve animals]. Relative blood flow in naïve and PBS-treated controls was similar to that for AdNull-treated animals. The increase in the

relative blood flow in AdVEGF-treated animals compared to controls, calculated as the ratio of microspheres/gm tissue in the ligated hindlimb versus microspheres/gm tissue in the contralateral control hindlimb, resulted
5 from an increase in the number of microspheres in the ligated hindlimb, not a decrease in the number of spheres in the contralateral control hindlimb. To confirm that there was adequate mixing of color microspheres during injection, relative blood flow to each hindlimb was
10 quantified in a separate group of animals without undergoing any iliac or femoral ligation (n=6). Relative blood flow (left versus right) in these animals was $99\% \pm 7\%$.

Angiography demonstrated significantly greater
15 vascularity in the ligated hindlimb in AdVEGF-treated animals than in controls, with collateral formation that partially reconstituted the distal hindlimb vasculature. The angiographic vessel score in the ligated hindlimb of AdVEGF-treated animals was significantly greater than
20 that of naïve, PBS-treated and AdNull treated controls. The number of angiographically visible collateral vessels in the naïve and PBS-treated animals were similar, as were the number of vessels in the PBS and AdNull treated animals. The number of vessels in the naïve control were
25 greater than that in the AdNull group, but both were less than the AdVEGF group.

Histologic evaluation of vascularity in adipose tissue and skeletal muscle was consistent with the observations made of relative blood flow and angiographic
30 evidence of enhanced vascularity. Compared to the naïve, PBS-treated and AdNull-treated controls, a significantly greater number of small blood vessels were observed 21 days following vector administration in the AdVEGF injected adipose tissue. Quantitative assessment of the
35 histologic samples of AdVEGF-treated adipose tissue resulted in a $52\% \pm 6\%$ increase in small vessel number when compared to the naïve, PBS and AdNull controls.

Similarly, histologic evaluation of AdVEGF-treated quadriceps skeletal muscle demonstrated a significantly greater number of capillaries per muscle fiber 21 days following vector administration compared to the naïve, PBS-treated and the AdNull-treated controls. Quantitative assessment of AdVEGF-treated skeletal muscle demonstrated a significant increase in mean capillary numbers per muscle fiber compared to naïve, PBS-treated and AdNull-treated controls.

The results demonstrate that adenoviral-mediated transfer of the DNA of human VEGF₁₆₅ in vivo to adipose and skeletal muscle tissues surrounding and distal to a site of vascular occlusion induces an angiogenic response adequate to attenuate the ischemia caused by subsequent acute vascular occlusion. Also, the results indicate that not only can angiogenic mediators be used to enhance angiogenic responses following an acute ischemic event, but they also can be used to "salvage" ischemic tissues threatened by subsequent acute vascular occlusion. The demonstration of enhanced blood flow to protect ischemic tissues weeks after the expressed angiogenic protein is no longer detectable illustrates that administration of an adenoviral vector comprising a DNA which encodes an angiogenic peptide is sufficient to provide a physiologically significant angiogenic response.

Example 4

This example illustrates the present invention's ability to improve myocardial perfusion and function in ischemic heart via adenoviral-mediated gene transfer of a DNA encoding an angiogenic peptide (particularly VEGF₁₂₁).

A model of chronic myocardial ischemia was created in Yorkshire swine weighing 28 to 30 kg. Animals were sedated with intramuscular tiletamine and zolazepam (Telazol, 3.3 mg/kg) and xylazine (0.10 mg/kg) and then intubated, and sedation was maintained with 0.5% to 2.0% isoflurane. A limited left thoracotomy was performed in

a sterile fashion through the fifth intercostal space, and a small incision was made in the pericardium. A 2.5 mm internal diameter ameroid constrictor (Research Instruments & MFG, Corvallis, Ore.) was placed around the circumflex artery as proximally as possible. Topical lidocaine 1% solution was applied to the circumflex artery at the ameroid constrictor site to prevent coronary artery spasm. The pericardium and chest were then closed, and the animal was allowed to recover.

The replication-deficient vector AdVEGF₁₂₁ is an E1a⁻, partial E1b⁻, partial E3⁻ adenoviral vector that contains an expression cassette in the E1 position (right to left) containing the cytomegalovirus (CMV) immediate early promoter/enhancer, an artificial splice sequence, the human VEGF₁₂₁ DNA, and the SV40 polyA/stop signal. AdNull (similar to AdVEGF₁₂₁, but with no gene in the expression cassette) was used as a control vector. All adenoviral vectors were propagated and titrated in 293 cells, purified by cesium chloride density purification, dialyzed, and stored at -70 °C. The viral stocks were demonstrated to be free of replication-competent wild-type adenoviruses. Biologic activity of the VEGF₁₂₁ transgene product was confirmed by demonstrating proliferation of human umbilical vein endothelial cells using [³H]thymidine incorporation, and *in vivo* confirmation of transgene expression was determined by enzyme-linked immunosorbent assay analysis of myocardial biopsy specimens obtained from AdVEGF₁₂₁ injection sites 3 days after vector administration.

Three weeks after ameroid constrictor placement, the left thoracotomy was reopened, and administration of the therapeutic vector, AdVEGF₁₂₁, or the control vector, AdNull, was performed by direct myocardial injection. Each vector was injected at 10 sites, each in 100 µl phosphate-buffered saline solution, pH=7.4, in the circumflex distribution (10⁸ pfu/injection). Pacing wires were placed in the left atrial appendage and tunneled

subcutaneously for subsequent stress ^{99m}Tc -labeled sestamibi assessment of regional myocardial perfusion by single photon emission computed tomography (SPECT) and echocardiographic assessment of regional wall thickening.

5 Regional myocardial perfusion was evaluated during rest and stress 3 weeks and 7 weeks after placement of the ameroid constrictor by means of ^{99m}Tc -sestamibi SPECT. During rapid atrial pacing at 200 beats/minute, animals received intravenous injections of a 5 mCi bolus of ^{99m}Tc -
10 sestamibi, and pacing was continued for approximately 3 minutes. The animals were then placed in the prone position in an ADAC Vertex dual detector gamma camera system (ADAC Laboratories, Milpitas, CA). A nongated SPECT study was then acquired in a "step-and-shoot" mode
15 over a 180-degree body-contouring orbit. The animal was allowed to return to baseline heart rate and then received an injection of a 25 mCi bolus of ^{99m}Tc -sestamibi before obtaining a rest SPECT, acquired in an analogous fashion.

20 The rest and stress SPECT studies were processed in a blinded fashion with the use of an integrated ADAC Pegasys computer. Stress and rest circumferential count profiles (polar plots) at the midventricular level were constructed by dividing the midventricular short-axis
25 image into 60 angular segments centered on the ventricular cavity, determining the number of counts per segment, normalizing the number of counts in each segment to the segment with the maximum number of counts (assigned a reference value of 100), and plotting the
30 normalized counts per segment versus the angular position of the segment. The polar plots were transferred to ASCII files for further analysis with the program SIGMAPLOT (Jandel Scientific, Corte Madera, CA).

35 For each animal, the extent of myocardial ischemia ("area") was determined from the difference between the rest and stress polar plots. The maximum severity of ischemia ("ischemia maximum") in the circumflex

distribution was determined by ascertaining the point of greatest difference between the rest and stress plots and measuring the difference in the plots at that point. The percent improvement in myocardial perfusion for each

5 animal was calculated for these two parameters as
("parameter" at 3 weeks - "parameter" at 7 weeks X
100)/("parameter" at 3 weeks).

Baseline regional myocardial function was assessed by echocardiography at rest and during stress at the time
10 of vector administration. Animals were sedated and placed in the left lateral decubitus position, and standard two-dimensional and M-mode transthoracic images were obtained with an HP2500 echocardiographic machine and a 3.0/3.5 MHz dual-frequency transthoracic transducer
15 (Hewlett-Packard, Andover, Mass.). From the right parasternal approach, short-axis, midpapillary views were obtained at rest for 3 minutes. The animals then underwent rapid left atrial pacing in a stepwise fashion to the target ventricular rate of 200 beats/min, at which
20 time imaging was recorded for an additional 3 minutes.

Regional wall thickening was determined by a single experienced investigator in a blinded fashion, tracing the endocardial and epicardial surfaces of the left ventricle in both diastole and systole using a Digisonics
25 CardioRevue System (Digisonics Inc, Houston, Tex.). Systolic wall thickening in each of six equal radial 60-degree segments was defined as mean systolic wall thickness minus mean diastolic wall thickness. Fractional wall thickening was calculated as mean
30 systolic wall thickening divided by mean diastolic wall thickness. The ischemic and nonischemic zones for each animal were defined from rapid atrial pacing images at 3 weeks (baseline ischemia) as the two contiguous segments with the lowest and highest fractional wall thickening,
35 respectively. This corresponded in all cases with the circumflex region and the septum, respectively. The same

zones for each animal were analyzed in rapid atrial pacing images at 7 weeks.

When each animal was put to death (4 weeks after vector administration), the heart was arrested with 40 mEq of KCl and then perfusion-fixed at 100 mm Hg with 1 l of McDowell-Trump fixative (4% formaldehyde, 1% glutaraldehyde, 1% NaH_2PO_4 , and 0.3% NaOH adjusted to pH=7.2). Ex vivo coronary angiography was performed by the same angiographer in a blinded fashion using a 5F end-hole wedge balloon catheter (Arrow Inc., Reading, Pa.) placed in the left main coronary artery. By means of cinefluoroscopy in the standard right anterior oblique projection with continuous image acquisition, 5 ml of contrast medium (Hypaque-76, Nycomed Inc., New York, N.Y.) was injected at a continuous rate until the entire left anterior descending coronary artery and its branches were completely opacified. Collateral vessels from the left anterior descending coronary artery, which reconstituted the circumflex coronary artery or obtuse marginal branch of the circumflex coronary artery, were quantified by three blinded observers using the grading method of Rentrop and associates, as described in J. Am. Coll. Cardiol., 5, 587-592 (1985), as follows: 0 = no filling of collateral vessels; 1 = filling of collateral branches of the circumflex or obtuse marginal branch without visualization of the epicardial segment; 2 and 3 = partial or complete filling of the epicardial segment of the circumflex or obtuse marginal artery via collateral vessels, respectively.

After angiography, the left ventricle of each heart was sectioned into three rings in the short axis. Forty 5 μm histologic sections from each heart were taken at equidistant intervals around the basal and midventricular rings, processed through paraffin, and stained with hematoxylin and eosin. Histologic evidence of infarction and inflammation for each tissue section was graded by a pathologist blinded to treatment on a scale of 0 to 4 as

follows: 0 = none; 1 = one to three small areas involved; 2 = less than 10% section surface; 3 = more than 10% and up to 50% section surface; and 4 = more than 50% section surface.

5 All of the 19 animals studied (AdVEGF₁₂₁, n=9; AdNull, n=10) survived until put to death 7 weeks after placement of the ameroid constrictor, without clinical evidence of toxicity. At 3 weeks (i.e., before therapy),
10 four of the 19 pigs (AdVEGF₁₂₁, n=2; AdNull, n=2) had evidence of myocardial infarction in the circumflex region, as demonstrated by (1) a fixed defect (no difference between rest and stress) in the circumflex zone of the ^{99m}Tc-sestamibi SPECT images and (2) a thinned, akinetic posterolateral region of the left
15 ventricle in short-axis views during echocardiography at rest. Consistent with the ^{99m}Tc-sestamibi SPECT and echocardiography suggesting myocardial infarction 3 weeks after ameroid constrictor placement, the gross pathologic evaluation 4 weeks later showed myocardial scarring and
20 thinning of at least 25% of the total ventricular mass. All four pigs in this subgroup also had histologic evidence of large transmural infarction. On the basis of these data, these four animals were excluded from further analysis. Thus the group of animals evaluated for
25 efficacy of therapy included seven AdVEGF₁₂₁-treated animals and eight AdNull (control) animals.

In vivo expression of the AdVEGF₁₂₁ vector was confirmed by demonstrating local myocardial VEGF expression after myocardial injection of 10⁸ pfu of
30 AdVEGF₁₂₁ (n=3). Three days after administration of the vector, myocardial level were 0.75 ± 0.25 ng/mg protein.

Circumferential count profiles (polar plots) of ^{99m}Tc-sestamibi SPECT data from the midventricular level were used to quantify (1) the extent and severity of
35 ischemia ("area") and (2) the most severe ischemia ("ischemia maximum"). Circumferential plots of rest images obtained at 3 weeks typically demonstrated minimal

perfusion defects, compared with plots of stress (pacing) images, which revealed decreased perfusion in the posterolateral region, corresponding to the occluded circumflex coronary artery distribution. The ischemic area and ischemia maximum were characteristically unchanged from baseline in AdNull animals assessed 4 weeks after vector administration. In contrast, AdVEGF₁₂₁ animals demonstrated improvement in myocardial perfusion 4 weeks after vector administration, as demonstrated by decreases in the ischemic area and ischemic maximum compared with baseline. Corresponding changes were noted at the apical, midventricular, and basal levels.

The ischemic area was similar in both the AdVEGF₁₂₁ and AdNull control animals at the time of vector administration. In contrast, the ischemic area was significantly reduced at 7 weeks in the AdVEGF₁₂₁ animals compared with the AdNull animals. The "percent improvement" in the area of ischemia of each animal 4 weeks after vector administration, compared with baseline, was approximately 2.4-fold greater in the AdVEGF₁₂₁ animals than in the AdNull animals (75% \pm 6% versus 32% \pm 11%, respectively).

The ischemia maximum in the circumflex distribution was also the same for the AdVEGF₁₂₁ and AdNull control animals at 3 weeks. In contrast, 4 weeks after vector administration, the ischemia maximum was significantly decreased in the AdVEGF₁₂₁ animals than in the AdNull control animals. Similarly, the "percent improvement" in the ischemia maximum was 2.5-fold greater in AdVEGF₁₂₁ animals than in the AdNull control animals (56% \pm 8% versus 22% \pm 6%).

Three weeks after ameroid constrictor placement, myocardial function in the ischemic circumflex region compared with the nonischemic septum was similar in the AdVEGF₁₂₁ group compared with AdNull controls as assessed by fractional wall thickening during rapid atrial pacing. In contrast, by 4 weeks after vector administration,

AdVEGF₁₂₁ treated animals demonstrated significantly greater improvement in fractional wall thickening during rapid atrial pacing than did AdNull control animals. Strikingly, contractile function in the circumflex segment of the AdVEGF₁₂₁ group approximated that of the septal (control) segment, as reflected by an ischemic minus nonischemic zone difference of "zero" in this analysis.

Ex vivo angiography performed 4 weeks after vector administration confirmed complete occlusion of the proximal circumflex coronary artery by the ameroid constrictor in all animals. AdNull treated animals characteristically demonstrated only partial filling of the obtuse marginal and circumflex coronary arteries. In contrast, animals that received AdVEGF₁₂₁ typically demonstrated nearly complete reconstitution of both the obtuse marginal and circumflex coronary circulations.

The collateral grade for the obtuse marginal and circumflex coronary arteries was significantly greater in the AdVEGF₁₂₁ animals than in the AdNull animals. Finally, the total number of angiographically visible collateral vessels filling the circumflex and obtuse marginal arteries was significantly greater in the AdVEGF₁₂₁ animals than in the AdNull animals.

The myocardium in 13 of the 15 animals in the study was available for assessment of inflammation (AdVEGF₁₂₁, n=5; AdNull, n=8). Minimal inflammation was detected in the myocardium of these animals evaluated 4 weeks after therapy, with no difference in the extent of inflammation between the AdVEGF₁₂₁ and AdNull groups (overall intensity score 0.3 ± 0.06 versus 0.4 ± 0.08).

The results indicate that adenoviral-mediated transfer of the DNA of human VEGF, specifically VEGF₁₂₁, directly into the myocardium of a mammal, as demonstrated with Yorkshire swine, with an occluded circumflex coronary artery results in significant and physiologically relevant improvement in regional

myocardial perfusion and contractile function during stress-induced myocardial ischemia. Importantly, this improvement was associated with increased myocardial collateral vessel development, "biologically bypassing" the experimentally occluded coronary artery segment.

Example 5

This example illustrates the present invention's ability to administer AdVEGF_{121.10} to the cardiac tissue of a human.

The replication-deficient vector AdVEGF_{121.10} is an E1a⁻, partial E1b⁻, E3⁻ adenoviral vector that contains an expression cassette in the E1 position containing containing the cytomegalovirus (CMV) immediate early promoter/enhancer, an artificial splice sequence, the human VEGF₁₂₁ DNA, and the SV40 poly A/stop signal. The AdVEGF_{121.10} vector was manufactured according to procedures used to construct and produce Ad_{cv}CFTR.10 and Ad_{cv}CD.10 for two human clinical trials (i.e., U.S. FDA Clinical Trials BB-IND 5702 and BB-IND 6442). Following production of AdVEGF_{121.10}, the vector was purified and stored at -70 °C with a titer of between 2x10⁹-2x10¹⁰ pfu/ml (i.e., 2x10¹¹-2x10¹² pu/ml assuming 100 particle units/pfu) in a carbohydrate-salt solution.

An open chest cardiac bypass surgical procedure with routine cardiopulmonary bypass was carried out with the administration of the AdVEGF_{121.10} vector following completion of the bypass procedure. The AdVEGF_{121.10} vector was administered directly to the myocardium during open chest cardiac surgery using an insulin syringe with a 28 gauge needle. Two human patients, who were candidates for routine coronary bypass surgery and have diffuse or non-bypassable disease in at least one other coronary artery distribution, were given AdVEGF_{121.10} administrations. For each of the two patients, the total dose (1x10⁷ pfu, 1x10⁹ pu) of the vector was divided into 10 aliquots (100 µl/aliquot), with each aliquot

administered to a site separated by 1.5-2.0 cm at a depth less than or equal to 5 mm.

The surgical procedures followed were such that the two patients fasted from food and liquids after midnight prior to the surgical procedure. A standard procedure was used by the cardiac anesthesiologist to prepare each individual and throughout the procedure. The individuals were transported to the operating room where standard monitors (three lead-modified EKG, pulse oximeter) were applied; premedications were administered as needed. Under local anesthesia, a radial arterial line was inserted for continuous blood pressure monitoring and blood sampling. Anesthesia was induced with midazolam, fentanyl, and/or thiopental. Supplemental anesthesia included midazolam, fentanyl, and/or thiopental, as well as isoflurane. Muscle relaxation was achieved with pancuronium. Succinylcholine was administered, as needed, for the facilitation of endotracheal intubation in which case an additional intravenous access was inserted. A central line (either internal jugular or subclavian vein) was inserted. Through that central line a pulmonary catheter was inserted to allow for monitoring of heart function (central venous pressure, pulmonary artery pressure, pulmonary artery wedge pressure, and computerized, thermodilution-calculated cardiac output). A transesophageal echocardiography probe was inserted to identify intracardiac structures and to assess valvular and ventricular function. A urinary bladder catheter was placed to measure urinary output. Anesthesia was administered at doses which permitted extubation within four to six hours upon conclusion of the procedure. The patients' chest was then prepped and draped in a standard sterile fashion using a betadine/alcohol solution. A small (approximately 0.5 mm radius, the same as a skin punch biopsy) skin biopsy was taken at the site of the skin incision. The biopsy was used for growing

autologous fibroblasts for assessment of cytotoxic T-lymphocytes directed against the adenoviral vector.

A median sternotomy was performed and the saphenous vein, or another relevant conduit, was harvested.

- 5 Following median sternotomy, aortic and right atrial cannulation was performed after heparin administration (3 mg/kg). The left internal mammary artery was identified and dissected from the chest wall. The patient was placed on cardiopulmonary bypass as per standard
- 10 practice. The cardiopulmonary bypass circuit included a Cobe Excel membrane oxygenator (Cobe Laboratories Inc., Lakewood, Colorado) and either a Cobe roller pump or a Biomedicus centrifugal pump (Biomedicus, Eden Prairie, MN). The circuit was primed with approximately 2200 ml
- 15 of crystalloid solution (200 ml of 25% albumin, 0.5 mg/kg mannitol, 1800 ml Ringer's lactate). The coronary arteries to be bypassed were identified and marked. Following crossclamping of the aorta, intermittent anterograde and/or retrograde cold blood cardioplegia
- 20 with moderate systemic cooling (28-32 °C) was used. A myocardial temperature probe was placed to continuously monitor myocardial temperature during cardioplegic arrest, and the systemic temperature was monitored using a bladder temperature probe. Distal anastomoses to the
- 25 coronary arteries using the reverse saphenous vein segments was performed, using running 7-0 Prolene sutures. The internal mammary artery was anastomosed in a similar fashion to the left anterior descending artery. The aortic crossclamp was removed, and the patient was
- 30 systemically rewarmed to 36 °C, utilizing the heat exchanger of the cardiopulmonary bypass circuit. Following initiation of rewarming, a partial aortic occlusion clamp was placed on the aorta. The sites of proximal anastomoses of the saphenous vein grafts were
- 35 marked on the aorta, and 4.8 mm pledgets were excised from the aortic root. The proximal portion of each

saphenous vein graft was anastomosed to the aorta using 6-0 Prolene sutures.

After completion of the proximal anastomoses, the 10 injections (100 μ l/injection) of AdVEGF_{121.1.1} were
5 administered in an obstructed coronary artery territory not amenable to bypass. One patient had the vector administered to the right coronary artery distribution, and the second patient had the vector administered to the left ventricle. The patients were separated from
10 cardiopulmonary bypass. Protamine was administered to reverse heparin-induced anti-coagulation. The aortic and atrial cannulas were removed, and all cannulation sites were oversewn. Temporary ventricular pacing wires were placed. Thirty-six french thoracostomy tubes were placed
15 in the left pleural space and mediastinum to provide post-operative drainage as per standard protocols. The sternum was reapproximated and closed using #20 wire. The fascia were closed in two layers using running 0 Vicryl suture. The skin was reapproximated with skin
20 clips. A similar closure was employed upon the legs at the site of saphenous vein harvest.

All of the references cited herein, including patents, patent applications, and publications, are hereby
25 incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended
30 that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method for enhancing the level of perfusion of blood to a target tissue comprising:

5 administering, via multiple applications to said tissue, a dose of a pharmaceutical composition comprising (a) a pharmaceutically acceptable carrier and (b) an adenoviral vector comprising a DNA encoding an angiogenic peptide, such that said level of perfusion of blood to
10 said tissue is enhanced.

2. A method for treating a target tissue suffering from or at risk of suffering from ischemic damage comprising:

administering, via multiple applications to said
15 tissue, a dose of a pharmaceutical composition comprising (a) a pharmaceutically acceptable carrier and (b) an adenoviral vector comprising a DNA encoding an angiogenic peptide, such that said dose has a therapeutic or prophylactic effect on said tissue.

20 3. A method of inducing angiogenesis in a target tissue comprising:

administering, via multiple applications to said
tissue, a dose of a pharmaceutical composition comprising
(a) a pharmaceutically acceptable carrier and (b) an
25 adenoviral vector comprising a DNA encoding an angiogenic peptide, such that angiogenesis is induced in said tissue.

4. A method for inducing collateral blood vessel formation in a target tissue affected by or at risk of
30 being affected by a vascular occlusion comprising:

administering to said tissue a dose of a
pharmaceutical composition comprising (a) a
pharmaceutically acceptable carrier and (b) an adenoviral
vector comprising a DNA encoding an angiogenic peptide,
35 such that said adenoviral vector contacts a region including the source, the terminus, and an area therebetween for said collateral blood vessel formation.

5. The method of any of claims 1-4, wherein said target tissue is within a discrete organ.

6. The method of claim 5, wherein said discrete organ is the heart.

5 7. The method of claim 6, wherein said heart is a human heart.

8. The method of any of claims 1-7, wherein each of said multiple applications is administered for about 0.5-15 cm³ of said target tissue.

10 9. The method of any of claims 1-8, wherein all of said multiple applications are administered within about 10 minutes.

10. The method of any of claims 1-9, wherein said multiple applications are substantially simultaneous.

15 11. The method of any of claims 1-10, wherein said angiogenic peptide is selected from the group consisting of VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, and VEGF₁₈₉.

12. The method of any of claims 1-11, wherein said vector is deficient in at least one essential gene
20 function of the E1 region of the adenoviral genome.

13. The method of any of claims 1-12, wherein said vector is deficient in part of the E3 region

14. The method of any of claims 1-13, wherein said vector has at least a partial deletion of the E1a region,
25 at least a partial deletion of the E1b region, and at least a partial deletion of the E3 region.

15. The method of any of claims 1-14, wherein said vector is deficient in at least one essential gene function of the E4 region of the adenoviral genome.

30 16. The method of any of claims 1-15, wherein said vector has at least a partial deletion of the E1 region, at least a partial deletion of the E3 region, and at least a partial deletion of the E4 region.

17. The method of any of claims 1-16, wherein said
35 DNA is oriented from right to left in the adenoviral genome of said adenoviral vector.

18. The method of claim 17, wherein said DNA is positioned in the E1 region of the adenoviral genome.

19. The method of any of claims 1-18, wherein said tissue is affected by or at risk of being affected by a
5 vascular occlusion.

20. The method of any of claims 1-19, wherein said dose is administered ex vivo to said target tissue.

21. The method of any of claims 1-19, wherein said dose is administered in vivo to said target tissue.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/01638

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/19 A61K48/00 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAJANAYAGAM ET AL: "DELIVERY OF VEGF TO ISCHEMIC TISSUE USING ADENOVIRAL-MODIFIED AUTOLOGOUS ENDOTHELIAL CELLS" CIRCULATION, vol. 94, 15 October 1996, page 1-646 XP002064912	4-7, 11-21
Y	see abstract 3773	1-3,8-10
X	MÜHLHAUSER ET AL: "VEGF165 EXPRESSED BY A REPLICATION-DEFICIENT RECOMBINANT ADENOVIRUS VECTOR INDUCES ANGIOGENESIS IN VIVO" CIRCULATION RESEARCH, vol. 77, 1995, pages 1077-1086, XP002064913	4-7, 11-21
Y	see the whole document	1-3,8-10

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

Internat. of Application No

PCT/US 98/01638

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAGOVERN ET AL: "REGIONAL ANGIOGENESIS INDUCED IN NONISCHEMIC TISSUE BY AN ADENOVIRAL VECTOR EXPRESSING VASCULAR ENDOTHELIAL GROWTH FACTOR" HUMAN GENE THERAPY, vol. 8, no. 2, 20 January 1997, pages 215-227, XP002064914	4-7, 11-21
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X	MAGOVERN ET AL: "DIRECT IN VIVO GENE TRANSFER TO CANINE MYOCARDIUM USING A REPLICATION-DEFICIENT ADENOVIRUS VECTOR" ANNALS OF THORACIC SURGERY, vol. 62, 1996, pages 425-434, XP002064915	4-7, 11-21
Y	see the whole document	1-3,8-10
X	MÜHLHAUSER ET AL: "IN VIVO ANGIOGENESIS INDUCED BY RECOMBINANT ADENOVIRUS VECTORS CODING EITHER FOR SECRETED OR NONSECRETED FORMS OF ACIDIC FIBROBLAST GROWTH FACTOR" HUMAN GENE THERAPY, vol. 6, 1995, pages 11457-1465, XP002064916	4-7, 12-21
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Information on patent family members

International Application No

PCT/US 98/01638

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